

## REVIEW

# Follicular dendritic cells and apoptosis: Life and death in the germinal centre

ERNST LINDHOUT and CORNELIS de GROOT

*Cellular Immunology Group, Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands*

Received 20 July 1994 and in revised form 29 September 1994

### Summary

The germinal centre forms a specialized microenvironment thought to play a key role in the induction of antibody synthesis, affinity maturation of B cells and memory B cell formation. Clonal-expanded follicular B lymphocytes with mutated antigen receptors (centrocytes) have to be selected on the basis of their capacity to compete for binding to antigen held in limited amounts on the follicular dendritic cells. In this way, only high-affinity B cells are selected. Binding to a follicular dendritic cell is an unconditional prerequisite for centrocytes to survive. Cells that do not succeed in binding to a follicular dendritic cell die rapidly by apoptosis. Apoptosis is a common form of cell death characterized by the activation of an endonuclease culminating in nuclear destruction. The pathway by which apoptosis is triggered varies from cell type to cell type. However, for germinal centre B cells this process is still poorly understood.

### Follicular dendritic cells and germinal centre B lymphocytes

#### *General architecture of lymphoid follicles*

During immune responses to T cell-dependent antigens, germinal centres (GCs) develop in the follicles of secondary (peripheral) lymphoid organs (MacLennan & Gray, 1986; Wacker *et al.*, 1990). GC formation is a dynamic process during the humoral immune response that involves the interaction of functionally different types of cells. GC reactions become apparent three days after immunization and include a high rate of B cell proliferation leading to the characteristic appearance of germinal centres (Fig. 1). It is generally believed that GC reactions play a central role in the formation of memory B cells (Klaus *et al.*, 1980; Kunkl & Klaus, 1981; MacLennan & Gray, 1986; MacLennan *et al.*, 1990; Tew *et al.*, 1989). The major constituents of GCs are activated B cells (Fig. 2c), macrophages (tingible body macrophages), T lymphocytes (Fig. 2e) and follicular dendritic cells (FDCs) (Fig. 2, a and b) (Butcher *et al.*, 1982; Rouse *et al.*, 1982; Stein *et al.*, 1982).

GCs can be divided anatomically into two main compartments (Fig. 1) (Nieuwenhuis & Opstelten, 1984); a dark zone and a light zone. The dark zone is populated by rapidly dividing B cells (centroblasts) which are believed to enter the GC only after initial encounter with antigen in the change-over zone between the interfollicular T cell areas and the follicles (MacLennan & Gray, 1986; Gray, 1988; Freedman *et al.*, 1990; Tew *et al.*, 1990). The light zone contains non-dividing centrocytes (progeny of centroblasts) and a dense network of FDCs (Nossal *et al.*, 1968; Szakal & Hanna, 1968; Tew *et al.*, 1989, 1990; Gray & Leander, 1990; Kroese *et al.*, 1990; MacLennan *et al.*, 1990; Wacker *et al.*, 1990). FDCs are large non-lymphoid cells with elongated cytoplasmic extensions that form the framework of the germinal centre (Nossal *et al.*, 1968; Szakal & Hanna, 1968). FDCs are unique for B cell follicles and have the capacity to take up antigen as antigen–antibody or antigen–complement complexes and to hold these in an immunogenic form for long periods of time (Tew & Mandel, 1978, 1979; Klaus *et al.*, 1980; Kosco *et al.*, 1988; Szakal *et al.*, 1988). In the

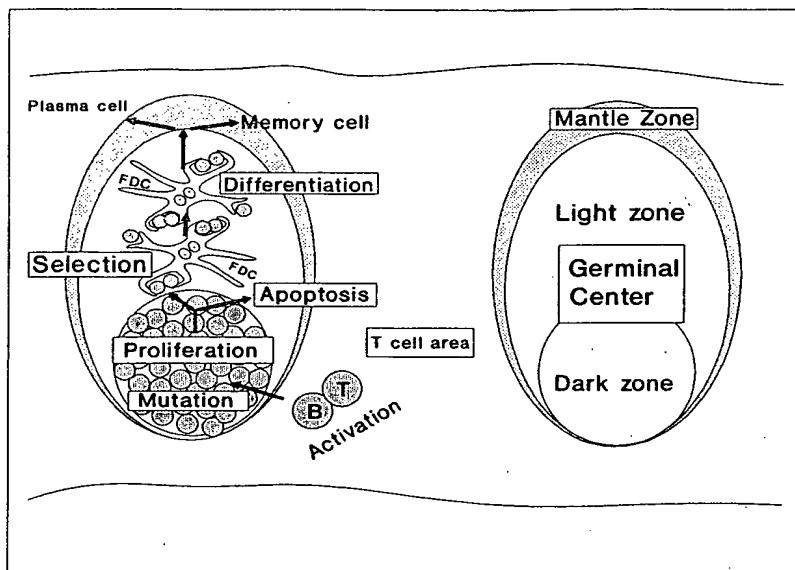


Fig. 1. Schematic drawing of a germinal centre. Antigen-specific B lymphocytes and T lymphocytes are activated by antigen somewhere in the T-B cell change-over area. Activated B cells locate into the follicle and start to proliferate. During this rapid proliferation somatic hypermutations take place in the genes encoding the variable part of the immunoglobulin heavy and light chains. After three to four days the follicle is filled with centroblasts and the characteristic germinal centre structure appears: a dark zone filled with proliferating centroblasts and a light zone filled with centrocytes (progeny of centroblasts) and a dense network of FDCs. It is believed that in the light zone selection of high-affinity B cells take place. Centroblasts entering from the dark zone must compete for binding to antigen on FDCs. Cells not succeeding in binding die rapidly by apoptosis. Cells binding to FDCs will differentiate further into memory cells or plasma cells.

light zone of GCs many centrocytes die *in situ* (Fliedner *et al.*, 1964). It was realized later that the cells died by apoptosis when Kerr *et al.* (1972) had described this process. However, not all centrocytes die, some centrocytes survive and leave the GC. It has been suggested that these cells become memory B cells (Coico *et al.*, 1983; Liu *et al.*, 1991a) or plasma cells (Liu *et al.*, 1991a). From studies *in vitro* it is known that isolated centrocytes degenerate rapidly by apoptosis upon culture at 37°C, and that apoptosis can be prevented by ligation of their surface immunoglobulin with immobilized antibodies (Liu *et al.*, 1989). Apoptosis in the light zone may reflect a process leading to the selection of only high-affinity B cells into the memory pool (Liu *et al.*, 1989). Cells that have undergone somatic mutation in their immunoglobulin

variable-region gene during proliferation in the dark zone, have to compete for binding to limited amounts of antigens presented by FDCs (MacLennan & Gray, 1986; Kosco *et al.*, 1988; Liu *et al.*, 1989). Consequently, those cells that manage to bind antigen due to the high affinity of their antigen receptors are presumably rescued from apoptosis by receiving positive signals of unknown nature (MacLennan & Gray, 1986; Kosco *et al.*, 1988; Lindhout *et al.*, 1993).

#### The follicular dendritic cell

In 1927, Maximow was the first who has described follicular dendritic cells as 'a nonlymphoid population of embryonic nonphagocytic reticulum cells'. In later

Fig. 2. Micrographs of cryostat sections of human tonsils stained with antibodies against: the FDC-specific markers DRC-1, CD21, the B cell marker CD20, the NK cell marker CD57 (also specific for GC T cells), the adhesion molecule ICAM-1 and IgM representing immune complexes. (a) Part of a germinal centre (GC) stained with the FDC-specific antibody DRC-1 shows the densely stained FDC-network. Note the sharp border between GC and non-stained mantle zone (MZ).  $\times 240$ . (b) Small GC stained with antibodies against CD21 (CR2) shows the characteristic FDC-network.  $\times 200$ . (c) Tonsil stained with anti-CD20 shows densely stained GC-B cells (GC) and medium stained mantle zone B cells (MZ).  $\times 75$ . (d) Higher magnification of CD20 stained tonsil shows two GCs (GC) and the interfollicular T cell area (T) containing many B cells.  $\times 150$ . (e) GC stained with antibodies against the NK cell marker CD57 shows several GC T lymphocytes (►) (N.B. All GC T cells express this NK-cell marker;  $\times 75$ .) (f) Anti-ICAM-1 staining shows the stained FDC-network in two GCs (GC) and the intensely stained numerous blood vessels (→).  $\times 75$ . (g) Anti-IgM staining represents IgM-immune complexes localized on the cell membrane of FDCs.  $\times 100$ .

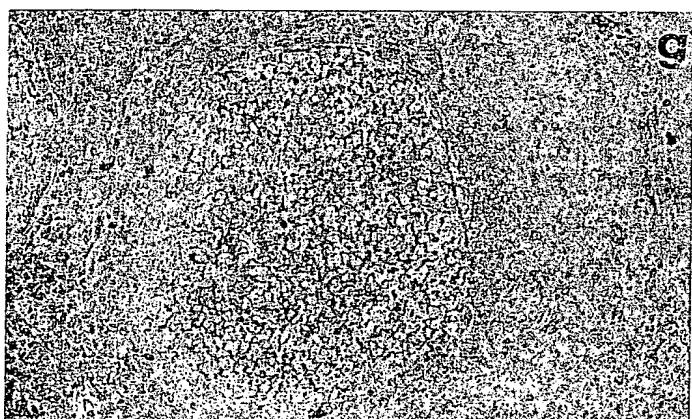
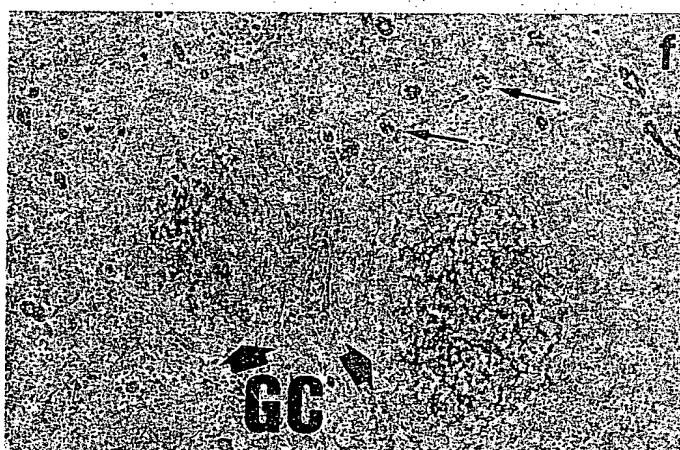
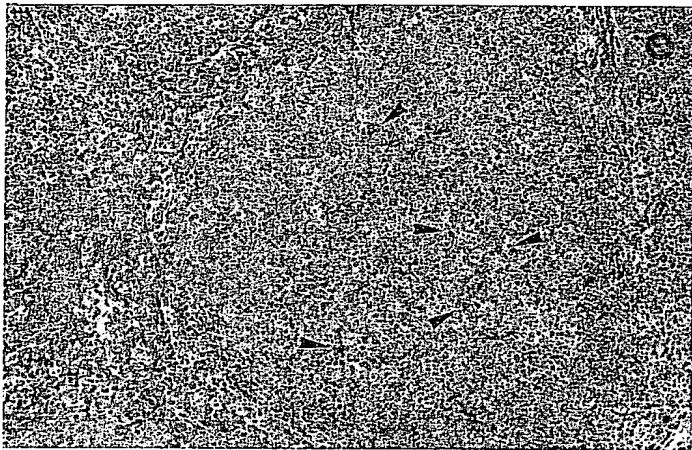
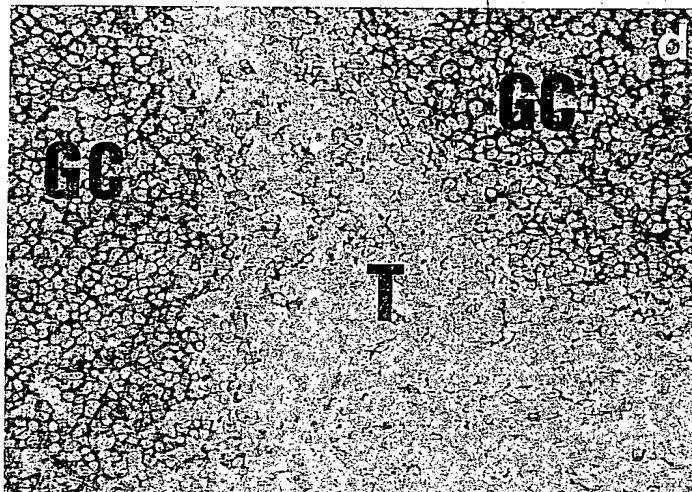
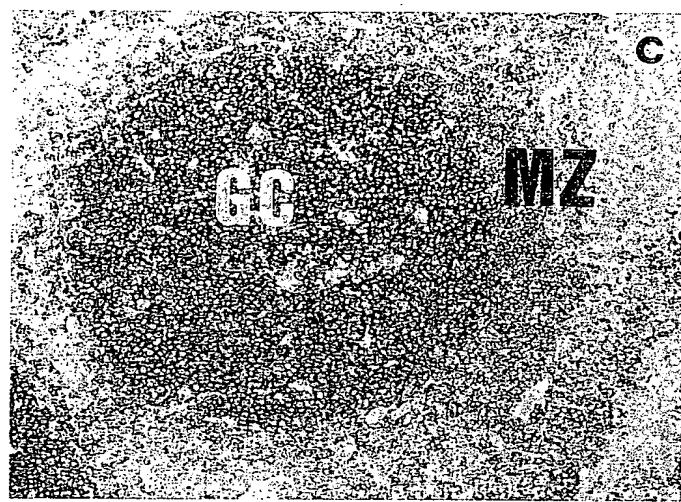
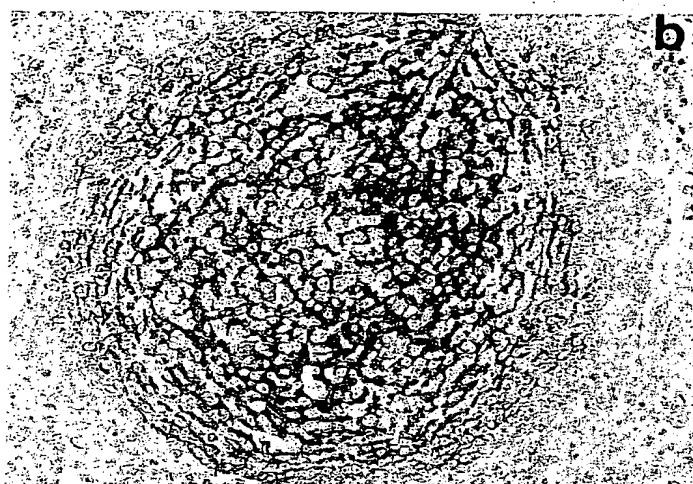
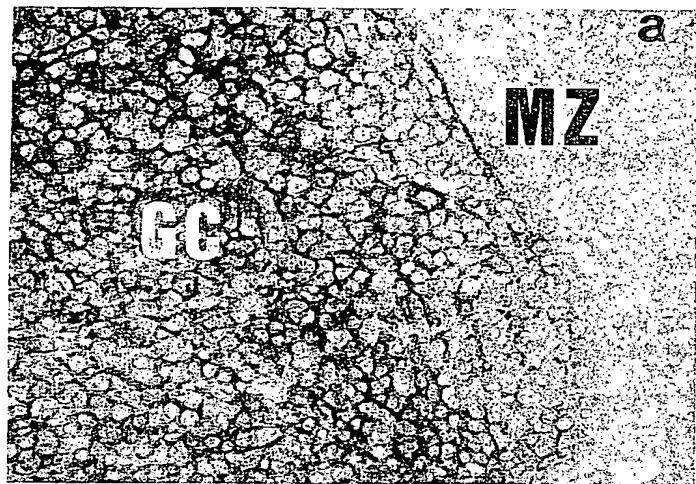


Fig. 2.

studies these cells were referred to as 'dendritic reticular cells or reticulum cells' (Nossal *et al.*, 1964), 'follicular dendritic reticulum cells', 'antigen-retaining cells', 'follicular antigen-binding dendritic cells' and 'dendritic macrophages' (Tew *et al.*, 1982). *In situ*, using mAb R4/23 (DRC-1) (Naiem *et al.*, 1983), it was demonstrated that FDCs form a dense three-dimensional network of long membrane processes between B cells of the follicle. FDCs are restricted to B lymphoid follicles (Fig. 2, a and b). Primary follicles contain only a few FDCs. Secondary follicles with well-developed germinal centres exhibit the dense three-dimensional network built up by the intercommunicating long, stretched cytoplasmic dendrites of FDCs, which sometimes appear to form syncytial complexes (Heinen *et al.*, 1991). The widely branched cytoplasmic processes result in marked enlargement of the cell surface which serves antigen fixation and presentation (Nossal *et al.*, 1968). It is presently unknown how FDCs develop from the few FDCs present in primary follicles to the dense network present in germinal centres. It is hardly possible to detect the exact outline of the cytoplasm of FDCs by both light microscopic as well as electron microscopic examination of lymphoid follicles. FDCs possess slenderly long, stretched dendrites sometimes developed only as membrane duplicatures. The cytoplasm around the nucleus consists of a narrow rim engulfing mostly two characteristic egg-shaped nuclei, with a small centrally localized distinct nucleolus. Since it is difficult to detect the cytoplasmic contours of FDCs *in situ*, these cells are best identified based on their typical nuclei (Fig. 3a). Also *in vitro*, isolated FDCs can easily be identified by their characteristic nuclei (Fig. 3b).

Despite their name FDCs are unrelated to other types of dendritic cells, including interdigitating cells (IDCs) found in T cell areas, Langerhans cells (LC) and dermal dendritic cells found in the skin and dendritic cells isolated from peripheral blood. FDCs are non-phagocytotic cells: they lack phagosomes in their cytoplasm, and the typical phagocyte enzyme lysozyme. In addition, FDCs do not have typical Birbeck granules in their cytoplasm which distinguish them from LC (see for review, Teunissen, 1992). Furthermore they have a unique phenotype (Gerdes *et al.*, 1983; Heinen *et al.*, 1984; Schriever *et al.*, 1989; Sellheyer *et al.*, 1989; Petrasch *et al.*, 1990; Tsunoda *et al.*, 1990; Koopman *et al.*, 1991; Parmentier *et al.*, 1991; Schriever *et al.*, 1991; Lindhout *et al.*, 1994), clearly different from that of other dendritic cells (Hart & McKenzie, 1988; Freudenthal & Steinman, 1990; King & Katz, 1990; Steinman, 1991; Teunissen, 1992; Lenz *et al.*, 1993; Nestle *et al.*, 1993) (Table 1).

As shown in Table 1, FDCs share some of their cell surface antigens with other dendritic cells, but differ in the expression of several other cellular markers. All dendritic cell types express CD40, MHC class I and

class II antigens and the adhesion molecules ICAM-1 (CD54) and VLA-4 (CD49d). Expression of MHC II by FDCs is a strange case because FDCs are not able to process antigens and to synthesize MHC II molecules. It is thought that MHC II molecules are picked up by the FDCs from surrounding GC B cells (MacLennan & Gray, 1986; Gray *et al.*, 1991). It may be that the contradictory data on expression by FDCs of other T cell and B cell related antigens (like CD4, CD19, CD20 and CD22) are caused by the same mechanism of randomly picking up of surface molecules from surrounding cells. For instance, it has been shown that FDCs themselves cannot synthesize CD4 (Schriever *et al.*, 1991), but it was found on FDCs by Schriever *et al.* (1989) and Parmentier *et al.* (1991), and not found on FDCs by others (Gerdes *et al.*, 1983; Sellheyer *et al.*, 1989; Petrasch *et al.*, 1990; Tsunoda *et al.*, 1990).

In contrast to DCs, FDCs express all three receptors for the C3 component of complement (CR1(CD35), CR2(CD21) and CR3(CD11b)) (Reynes *et al.*, 1985; Schriever *et al.*, 1989; Sellheyer *et al.*, 1989; Petrasch *et al.*, 1990; Lindhout *et al.*, 1994) and the adhesion molecule VCAM-1 (CD106) (Schriever *et al.*, 1989; Petrasch *et al.*, 1990; Koopman *et al.*, 1991; Lindhout *et al.*, 1994). Also in contrast to DCs, FDCs have immunoglobulin (Ig) on their surface (as immune complexes) and also a set of FDC specific markers. Lack of expression of the leukocyte common antigen CD45 and of mRNA encoding for CD45 (Schriever *et al.*, 1989, 1991; Tsunoda *et al.*, 1990; Lindhout *et al.*, 1994) is another feature that distinguishes FDCs from DCs. In addition, absence of CD45 proves that FDCs are non-haematopoietic cells and that they are, therefore, not related to monocytes or macrophages. To delineate the derivation of FDCs, these cells are also tested for expression of non-haematopoietic cell markers. FDCs do not express the endothelial cell markers factor VIII and CD31 (Schriever *et al.*, 1989; Lindhout *et al.*, 1994), the muscle-specific marker desmin (Tsunoda *et al.*, 1990) and the epithelial marker epithelial membrane antigen (EMA) (Schriever *et al.*, 1989; Petrasch *et al.*, 1990) and also do not stain for cytokeratin (Petrasch *et al.*, 1990; Tsunoda *et al.*, 1990). Further elucidation of the origin of FDCs came from Tsunoda *et al.* (1990), who observed that FDCs contain vimentin, which forms specific intermediate filaments in mesenchymal-derived cells such as fibroblasts, chondrocytes and endothelial cells. These results were consistent with observations of Kojima *et al.* (1968) who described FDCs as mesenchyme-derived according to their ultrastructural morphology and of Villena *et al.* (1983) who showed that FDCs originate from stromal cells with a fibroblastic appearance. Recent results from our group (Lindhout *et al.*, 1994) show that FDC are positive for the fibroblastic marker MAS516, supporting the idea that FDCs are mesenchyme-derived.

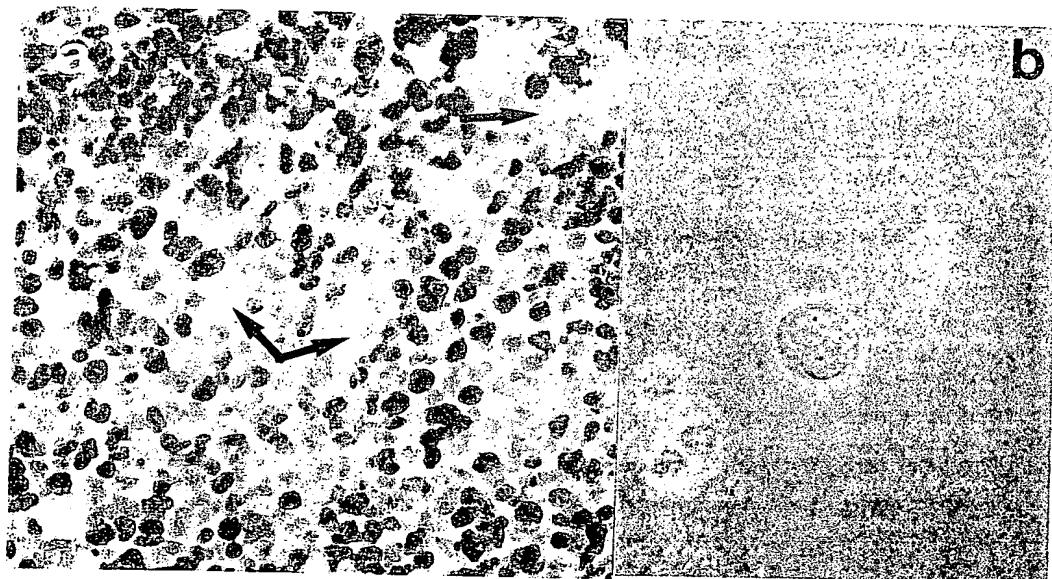


Fig. 3

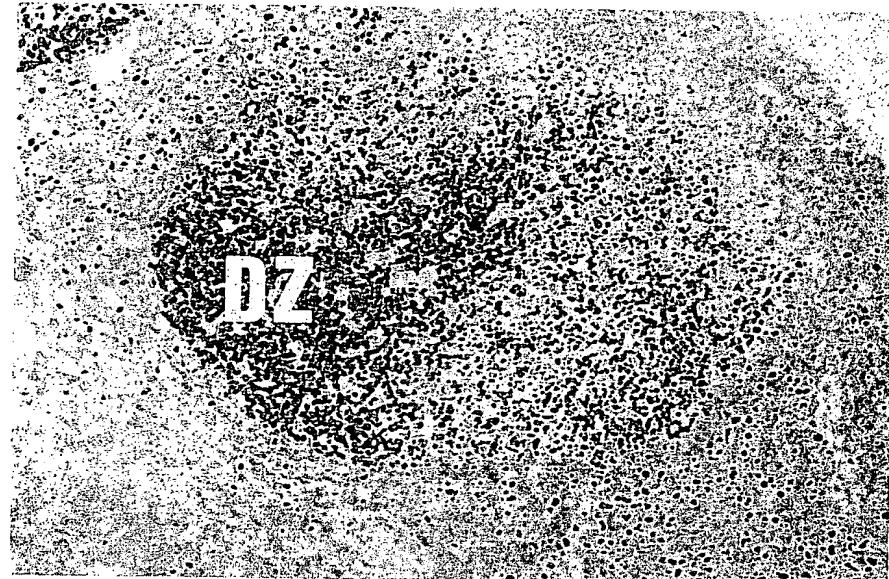


Fig. 4

**Fig. 3.** FDCs with characteristic egg-shaped nuclei and distinct nucleoli. (a) High magnification of cryostat section shows FDCs in the GC recognizable by the typical nuclei and centrally located nucleoli (→).  $\times 300$ . (b) Micrograph of an isolated FDC with characteristic egg-shaped nuclei.  $\times 640$ .

**Fig. 4.** Micrograph of a GC stained with the proliferation marker Ki-67 shows numerous proliferating centroblasts forming the dark zone of a GC (DZ).  $\times 66$ .

#### Antigen trapping by FDCs

Some time after antigenic stimulation, antigens localize on the surface of FDCs. These antigens, trapped as immune complexes (ICs) can be retained for very long periods by the FDCs (Nossal *et al.*, 1964; Nossal & Ada, 1971; Szakal & Hanna, 1968; Szakal *et al.*, 1985, 1988; Straus, 1970; Klaus & Humphrey, 1977; Klaus *et al.*,

1980; Tew & Mandel, 1978, 1979; Tew *et al.*, 1980; Mandel *et al.*, 1981; Kosco *et al.*, 1988) (Fig. 2g). Binding of ICs to FDCs is complement-mediated (Klaus & Humphrey, 1977; Joling *et al.*, 1993), since IC-trapping can be completely abrogated using cobra venom factor (Klaus & Humphrey, 1977; Papamichail *et al.*, 1975). In addition, FDCs express all three complement receptors

Table 1. Phenotype of isolated follicular dendritic cells and other dendritic cell types

Antigen	Dendritic cell type				
	<i>FDC</i> <sup>1-10</sup>	<i>IDC</i> <sup>11-13</sup>	<i>LC</i> <sup>14,15</sup>	<i>DC</i> <sup>13,16</sup>	<i>DDC</i> <sup>15,17</sup>
<b>T cell</b>					
CD1a	-	-	+	-	+
CD1b	-	-	-	-	-
CD1c	-	-	+	-	+
CD2	-	-	-	-	-
CD3	-	-	-	-	-
CD4	- <sup>a,4-8</sup> / + <sup>3,8</sup>	+/- <sup>b</sup>	+	-	-
CD6	-	-	-	-	-
CD7	-	-	-	-	-
CD8	-	-	-	-	+
<b>B cell</b>					
CD19(B4)	- <sup>3,7,9</sup> / + <sup>2</sup>	-	-	-	-
CD20(B1)	- <sup>a,3,7,10,18</sup> / + <sup>1</sup>	-	-	-	-
CD21(CR2)	+ <sup>a</sup>	-	-	-	-
CD22(BL-CAM)	- <sup>3,7</sup> / + <sup>9</sup>	-	-	-	-
CD23(IgERII)	+/- <sup>c</sup>	-	-	-	-
CD24(BA-1)	-	-	- <sup>14</sup> / + <sup>15</sup>	-	+
CD39	+	+	-	-	-
CD40	+	+	- <sup>14</sup> / + <sup>14,15</sup>	+	+
CD80(B7/BB1)	-	-	+	-	+
B5	-	-	-	-	-
PCA-1	-	-	-	-	-
<b>Myeloid</b>					
CD11b(CR3, C3bIgR)	+	-	+	-	+
CD11c(gp150/195)	-	-	+	+	+
CD13	-	-	-	-	+
CD14	+ <sup>1-4,10</sup> / - <sup>7</sup>	-	-	-	- <sup>15</sup> / + <sup>c17</sup>
CD15	-	-	-	-	-
CD16(Fc $\gamma$ RIII)	- <sup>3,4,9,10</sup> / + <sup>1</sup>	-	-	-	-
CD32(Fc $\gamma$ RII)	- <sup>d4,9,10</sup> / + <sup>1,3,9</sup>	-	+	-	+
CD33(gp67)	-	-	+	-	+
CD34(gp105/120)	-	-	-	-	-
CD35(CR1, C3bR)	+	-	-	-	-/(-) <sup>c</sup>
CD64(Fc $\gamma$ RI)	+ <sup>a</sup> / - <sup>b</sup>	-	-	-	-
<b>Adhesion</b>					
CD11a(LFA-1 $\alpha$ )	-	+	- <sup>14</sup> / + <sup>15</sup>	+	+
CD18( $\beta_2$ -integrin)	- <sup>9,10</sup> / + <sup>3</sup>	+	+	+	+
CD29( $\beta_1$ -integrin)	+	-	+	+	-
CD41(gpIb/IIIa)	-	-	-	-	-
CD49c(VLA-3 $\alpha$ )	+	-	+	-	-
CD49d(VLA-4 $\alpha$ )	+	-	+	-	-
CD49e(VLA-5 $\alpha$ )	+	-	+	-	-
CD49f(VLA-6 $\alpha$ )	+	-	+	-	-
CD54(ICAM-1)	+	+	+	+	+
CD1064(VCAM-1)	+	-	-	-	-
<b>Non Lineage</b>					
CD5	-	-	-	-	+
CD9	-	-	-	-	-
CD10(CALLA)	-	-	-	-	-
CD25(IL2R $\alpha$ )	-	+/-	-	-	+ <sup>c</sup>
CD30	-	+	-	-	-
CD37	+	-	-	-	-
CD44(PgP-1)	-	-	-	-	-
CD45(LCA-1)	- <sup>a,6,7,9,10</sup> / + <sup>1</sup>	+	+	+	+

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Table 1—continued

Antigen	Dendritic cell type				
	<i>FDC</i> <sup>1-10</sup>	<i>IDC</i> <sup>11-13</sup>	<i>LC</i> <sup>14,15</sup>	<i>DC</i> <sup>13,16</sup>	<i>DDC</i> <sup>15,17</sup>
CD71(TrfR)	+	—		—	
CDw75	+				
HLA-I	+	+	+	+	+
HLA-II	+	+	+	+	+
<b>FDC-associated</b>					
DRC-1	+	—			
KiM4	+				
12B1	+				
BU10	+				
BU30	+				
<b>Other</b>					
sIgM	+ <sup>1,2,4,5,8,10</sup> / <sub>—</sub>	—			
sIgG	+ <sup>1,2,4,5,8,10</sup> / <sub>—</sub>	—			
sIgA	+ <sup>2,4,5</sup> / <sub>—</sub>	—			
sIgD	+ <sup>1,2,4,5</sup> / <sub>—</sub>	—			
S-100	—		+		
CD56(NKH-1)	—	—			
Lysozyme	—				
Factor-VIII	—				
CD31(PECAM-1)	—				
EMA	—				
Birbeck granule	—	—	+	—	—
C3b	+				
C1q	+				
CK1	—				
TdT	—				
MAS516	+				
BCL-2	—				
Ki67	—				
LMP-1	—				
EBNA-2	—				

<sup>a</sup> PCR study.<sup>b</sup> + with Leu3, — with OKT4.<sup>c</sup> Subset is positive.<sup>d</sup> (4x) staining with four different monoclonal antibodies.

FDC = follicular dendritic cell; IDC = interdigitating cell; LC = Langerhans cell; DC = dendritic cell; DDC = dermal dendritic cell; EMA = endothelial membrane antigen; TdT = terminal deoxynucleotidyl transferase; CK1 = cytokeratin; PCA-1 = plasma cell antigen 1; MAS516 = human fibroblast marker.

Open space means not done.

References 1. Petrasch *et al.* (1990); 2. Heinen *et al.* (1984); 3. Schriever *et al.* (1989); 4. Sellheyer *et al.* (1989); 5. Gerdes *et al.* (1983); 6. Schriever *et al.* (1991); 7. Tsunoda *et al.* (1990); 8. Parmentier *et al.* (1991); 9. Koopman *et al.* (1991); 10. Lindhout *et al.* (1994); 11. Hart & McKenzie (1988); 12. King & Katz (1990); 13. Steinman (1991); 14. Teunissen (1992); 15. Lenz *et al.* (1993); 16. Freudenthal & Steinman (1990); 17. Nestle *et al.* (1993); 18. Szakal *et al.* (1985).

(Table 1). It is not clear whether receptors for immunoglobulin G (Fc $\gamma$  receptors) are involved in binding of ICs to FDCs because the presence of Fc $\gamma$  receptors on FDCs is questionable (Table 1, CD16, CD32 & CD64).

Trapping of antigen by FDCs as ICs is thought to play a crucial role in the generation of memory B lymphocytes during germinal centre reactions (Thor-

becke *et al.*, 1974; Klaus *et al.*, 1980), because it was found that generation of memory B cells appeared to be severely decreased by inhibiting IC-trapping (Klaus & Humphrey, 1977). The general idea is that antigen in ICs is needed for the selection of high-affinity B cells into the memory B cell pool (Klaus & Kunkl, 1981; MacLennan & Gray, 1986, 1989; Gray & Leanderson, 1990).

### FDC-B cell interactions.

As reviewed by MacLennan & Gray (1986), activation and differentiation of B cells by T cell-dependent antigens is thought to occur in two phases. Newly formed virgin B cells and B cells of the peripheral pool are activated with antigen in the changeover zone between the interfollicular T cell areas and the follicles (MacLennan & Gray, 1986; Gray, 1988; Freedman *et al.*, 1990; Tew *et al.*, 1990). Subsequently, certain activated B cells will differentiate into IgM-producing plasma cells while other activated B cells will localize in follicles consisting of small resting B cells and a network of FDCs. Activated B cells proliferate in the follicle (Fig. 4) and fill the whole FDC-network, pushing the small resting B cells away, which will form the mantle zone. One activated B cell is sufficient to start a GC-reaction but on average, newly generated GCs will develop from three precursor cells per GC (Kroese *et al.*, 1987; Liu *et al.*, 1991c). After 72 to 84 h the network has become completely filled with activated B cells ( $\pm 10^4$  cells), suggesting a cell cycle time in these B cells of 6 to 7 h (Zhang *et al.*, 1988; Liu *et al.*, 1991c). At this time point, changes occur within the follicle centre resulting in the appearance of the classical structure associated with fully-developed GC (Fig. 1) containing a dark zone and a light zone. The dark zone is populated by rapidly dividing centroblasts, which do not increase in number anymore but continually give rise to centrocytes. The light zone is populated by a dense network of FDCs (expressing high numbers of ICAM-1) (Hardie *et al.*, 1993) (Fig. 2f) and by non-dividing centrocytes (progeny of centroblasts).

It has been suggested that during the proliferation phase, somatic mutations of the rearranged Ig variable genes (Berek *et al.*, 1991; Jacob *et al.*, 1991) and their adjacent flanking regions (Gearhart & Bogenhagen, 1983) occur in the dark zone. At an estimated hypermutation rate of one per 1000 base pairs per generation (McKean *et al.*, 1984; Allen *et al.*, 1987; Berek *et al.*, 1987), nucleotide exchange in the variable region of the antibody gene will occur in nearly every division cycle. The rapid cell cycle of B cells within the GC leads to the generation of clones expressing a broad spectrum of variant antibody molecules. Random mutations inserted into the variable region of the antibody genes will only rarely lead to an improvement of the specificity for the antigen (Weigert, 1986). Despite this, the affinity of antibodies is found to increase 10-fold over a period of only two weeks (Huchet & Feldmann, 1973). On the basis of experi-

ments in mice (Griffiths *et al.*, 1984; Berek *et al.*, 1985), it was found that maturation of the immune response (= increase in antibody-affinity) is due to hypermutation and selection of high-affinity receptor variants (Griffiths *et al.*, 1984). Selection of these high-affinity B cells is thought to be a major function of FDCs. Limited amounts of undegraded antigen presented on the surface of FDCs in the form of ICs may determine which B cells are allowed to differentiate into memory cells. Experiments in mice showed that FDCs released ICs as immune-complex-coated bodies (iccosomes) that were endocytosed by GC B cells and subsequently processed and probably presented to T cells (Kosco *et al.*, 1988, 1989; Szakal *et al.*, 1988; Burton *et al.*, 1991; Terashima *et al.*, 1992). The formation and release of iccosomes would provide an alternative system for the delivery of antigen to, and the activation of antigen-specific B cells and T cells in the germinal centre, but as far as we know the formation of iccosomes has never been described for the human.

Selection of high-affinity B cells during germinal centre reactions is thought to take place in the light zone of germinal centres (MacLennan & Gray, 1986; MacLennan *et al.*, 1989, 1990; Gray & Leanderson, 1990; Leanderson *et al.*, 1992). Centroblasts (converting to centrocytes) enter from the dark zone and must compete for binding to antigen on FDCs. Those cells that do not succeed in binding will die rapidly through apoptosis. In the basal part of the light zone many cells are found with the characteristic features of apoptosis.

Binding of centrocytes to FDCs is mediated by interactions of the adhesion molecules ICAM-1 and VCAM-1 on the FDC and LFA-1 and VLA-4, respectively, on the centrocyte (Freedman *et al.*, 1990; Koopman *et al.*, 1991; Lindhout *et al.*, 1993). It was found *in vitro* that binding of B cells to FDCs, leading to the formation of typical FDC-B cell clusters (Fig. 5), is a rapid process depending on temperature (Louis *et al.*, 1989; Schriever *et al.*, 1989; Lindhout *et al.*, 1993) and presence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Louis *et al.*, 1989; Schriever *et al.*, 1989). Addition of antibodies against LFA-1 and/or VLA-4 could totally inhibit cluster formation (Lindhout *et al.*, 1993). Isolated GC B cells degenerate rapidly by apoptosis *in vitro* upon culturing at 37°C (Liu *et al.*, 1989). However, binding of GC B cells to FDCs *in vitro* prevents B cells from apoptosis (Lindhout *et al.*, 1993). GC B cells are also rescued from apoptosis *in vitro* by cross-linking the antigen receptor with immobilized antibodies (Liu *et al.*, 1989), by the addition of antibodies against CD40 or CD21 (Liu *et al.*,

Fig. 5. Phase-contrast micrograph of an FDC-B cell cluster *in vitro*. Isolated FDCs cultured for 16 h with GC B cells form spherical clusters containing on average one FDC and 10 B cells.  $\times 640$ .

Fig. 6. (a) Cryostat section of tonsil labelled with anti-BCL2 antibodies (mAb 124 (Pezzella *et al.*, 1990)) shows only BCL-2 positive cells outside the germinal centre.  $\times 150$ . (b) A higher magnification demonstrates the sharp border of stained mantle zone cells (MZ) and non-stained cells (GC).  $\times 300$ .

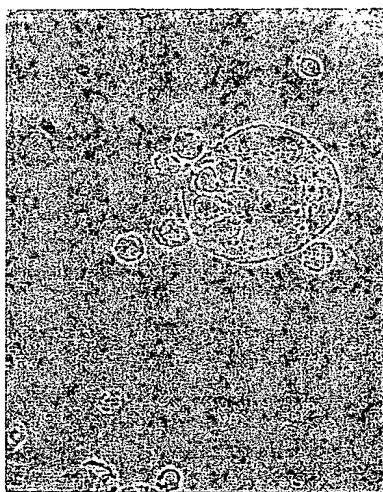


Fig. 5

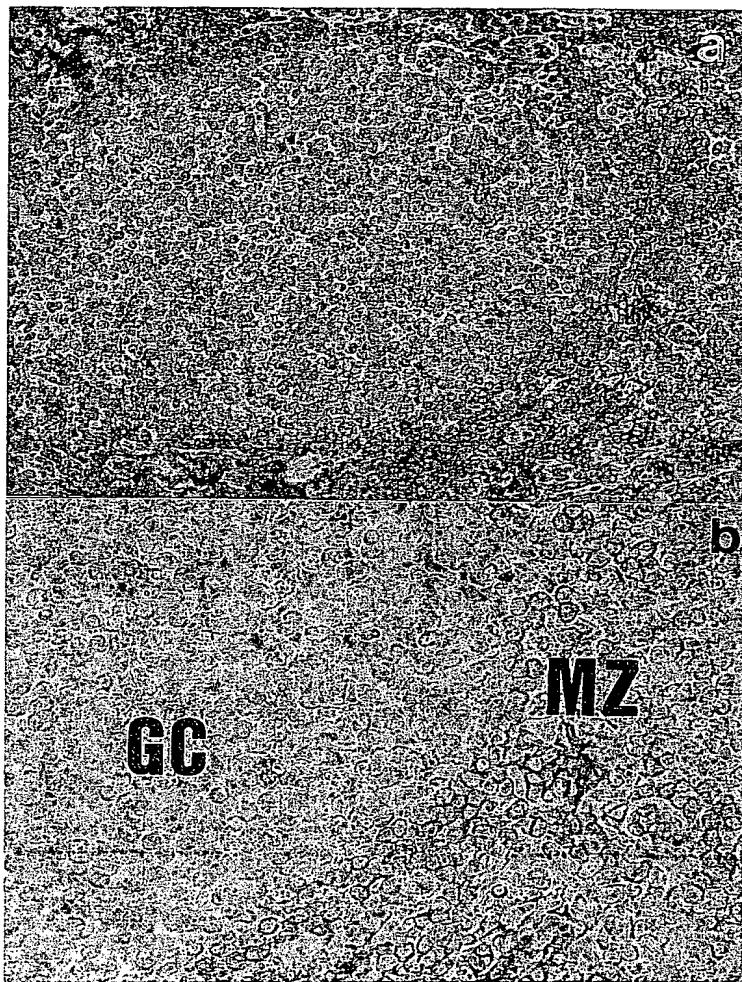


Fig. 6

al., 1989, 1991a; Bonnefoy *et al.*, 1993), by adding a combination of soluble CD23 and IL1 $\alpha$  (Liu *et al.*, 1991a), or by cross-linking of the adhesion molecules LFA-1 and/or VLA-4 using immobilized ICAM-1 or VCAM-1 respectively (Koopman *et al.*, 1994). Therefore, it can be concluded that rescue of GC B cells from apoptosis by binding to FDCs *in vivo* is most likely mediated by a combination of engaging antigen and ICAM-1 and VCAM-1. In contrast, experiments *in vitro* suggest that interaction with ICAM-1 and VCAM-1 alone is sufficient to prevent apoptosis in GC B cells (Lindhout *et al.*, 1993; Koopman *et al.*, 1994) and that signalling via the antigen receptor is not a prerequisite. However, it is difficult to believe that this reflects a physiological situation because it would bypass antigen-specific affinity selection. Hedman & Lundgren (1992) showed that upon cross-linking of antigen receptors on B cells, the high-avidity state of LFA-1 is induced within 5 min, resulting in rapidly binding to ICAM-1. There is probably a crucial synergistic effect between signals delivered through the antigen receptor and the adhesion receptors for effective selection of B cells *in vivo*. Low levels of antigen receptor cross-linking by low amounts of antigen held on FDCs (not sufficient for full B cell activation and definitive rescue from apoptosis (Liu *et al.*, 1989)) leads to activation of the adhesion molecules LFA-1 and VLA-4 on B cells (Dang & Rock, 1991; Hedman & Lundgren, 1992). Subsequently, GC B cells bind to ICAM-1 and VCAM-1 on the FDC in the basal part of the light zone and are then provided with signals required for their rescue from apoptosis and further differentiation. This differentiation process is thought to take place in the more apical part of the light zone (Fig. 1). In this zone the FDCs express large amounts of CD23 (Hardie *et al.*, 1993). In combination with IL1 $\alpha$ , CD23 can provide GC B cells with signals inducing some features of plasmablasts (Gordon *et al.*, 1989; Liu *et al.*, 1991a) and also prevent GC B cells from apoptosis by inducing *BCL-2* expression. The *BCL-2* gene encodes for a 26 kDa protein (Cleary *et al.*, 1986; Tsujimoto & Croce, 1986; Pezzella *et al.*, 1990) localized mainly in mitochondria (Hockenberry *et al.*, 1990) and cytosol (Hockenberry *et al.*, 1990; Liu *et al.*, 1991a), but it can also be found on the nuclear membrane and to a lesser extent on the cell membrane (Monaghan *et al.*, 1992; De Jong *et al.*, 1994). Whereas this protein is not expressed

in most germinal centre cells, it is found in virtually all extrafollicular B cells (Liu *et al.*, 1991b; Nunez *et al.*, 1991; Levy & Brouet, 1994) (Fig. 6). Expression of *BCL-2* shows to be important for cell survival (Hockenberry *et al.*, 1990; Henderson *et al.*, 1991; Liu *et al.*, 1991a, b; Nunez *et al.*, 1991; Danescu *et al.*, 1992; Garcia *et al.*, 1992; Milner *et al.*, 1992; Vaux, 1993). Signals provided by FDCs in the apical part of the light zone may induce expression of *BCL-2* resulting in centrocytes leaving the germinal centre as differentiated cells that no longer require signals from the microenvironment of GCs to survive.

### Apoptosis in germinal centre B cells

#### General features of apoptosis

Apoptosis is a form of cell death involving membrane blebbing, loss of cell volume, and chromatin margination along the nuclear envelope followed by collapse of the nucleus into fragments with very dense chromatin, yet still surrounded by the nuclear and the plasma membranes (Wyllie, 1988). An important difference between necrosis and apoptosis is that the rupture of cells occurring in necrosis releases cell contents that are chemotactic and induce inflammatory reactions to remove cell debris (Wyllie, 1988). Apoptotic cells retain their integrity or may break into membrane-enveloped apoptotic bodies, some of which contain chromatin. These are phagocytized by phagocytes (Wyllie, 1988). In germinal centres, apoptotic B cells are phagocytized by the so-called tingible body macrophages. Phagocytosis usually starts as soon as the process of apoptosis has begun and well before cells lose their membrane integrity and for example, take up vital dyes. In this way, an inflammatory reaction is not elicited. The hallmark of apoptosis is the collapse of the nucleus, while other organelles retain their structure relatively well (Kerr *et al.*, 1972; Wyllie, 1980). Chromatin becomes extremely condensed and tends to marginate to a crescent along the nuclear envelope; in many cell types chromatin collapses into its most dense form, i.e. one or several spherical fragments. The nuclear collapse during apoptosis reflects extensive damage to chromatin. Wyllie (1980) has demonstrated that during apoptosis chromatin is degraded into single and multiple

Fig. 7. Gel electrophoresis of DNA isolated from apoptotic cells. Lane 1: 100-bp marker; lane 2: DNA isolated from apoptotic cells showing the characteristic ladder pattern of 200 bp and multiples thereof.

Fig. 8. (a) May-Grünwald/Giemsa-stained cytopsin preparation of cultured GC B cells. Apoptotic cells can be clearly distinguished from viable cells by their condensed and fragmented nuclei. (►)  $\times 415$ . (b) Fluorescence micrograph of a culture of FDCs and GC B cells stained with Hoechst 33258. Viable and apoptotic cells can be distinguished on the basis of their different staining pattern. In viable cells (found only in FDC-B cell clusters), the staining pattern is found as a fine network of pale and euchromatic stained chromatin (→). In apoptotic cells the DNA appears as brightly stained (sometimes fragmented) nuclei with loss of structural details (►).  $\times 500$ .

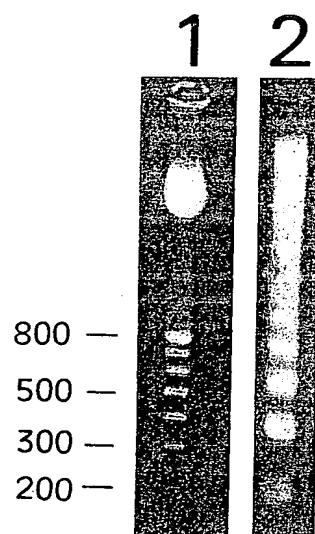


Fig. 7

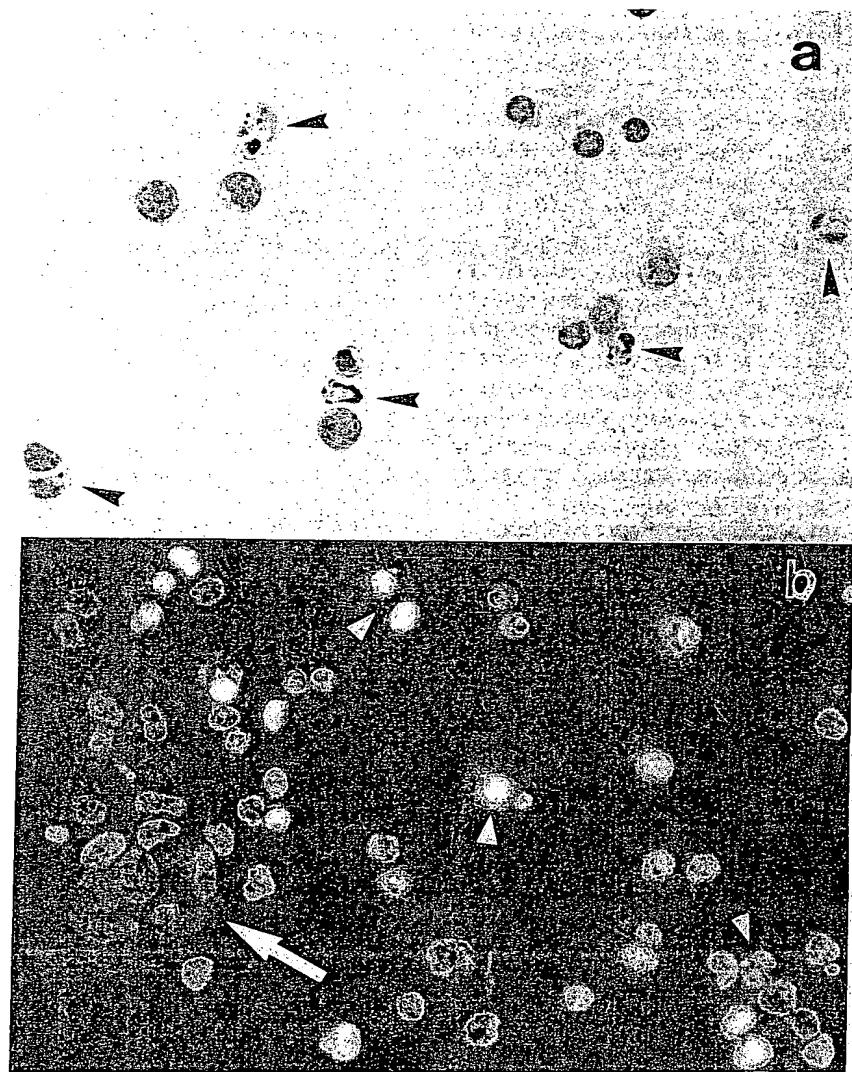


Fig. 8

nucleosomes. Because nucleosomes are spaced regularly at approximately 180–200 base pairs (bp) intervals and integral multiples thereof, electrophoresis of DNA from apoptotic cells reveals a ladder pattern of bands (Fig. 7). This ladder pattern is characteristic for apoptosis and is due to the action of an endonuclease that is found in the nuclei of certain cells. This endonuclease cleaves DNA in the inter-nucleosomal linker regions (Wyllie & Morris, 1982; Wyllie *et al.*, 1984; Arends *et al.*, 1990). There is no sequence specificity to this cleavage. Endogenous endonuclease can be activated in isolated nuclei in the presence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions (Cohen & Duke, 1984; Arends *et al.*, 1990). However, it is still questionable if there is endonuclease in the nuclei of most cell types or that *de novo* synthesis of endo-nuclease is required (Cohen *et al.*, 1985; Compton & Cidlowski, 1987; Ucker, 1987; Alnemri & Litwack, 1989; Nieto & López-Rivas, 1989).

#### *Induction of apoptosis*

Several agents have been described that can induce apoptosis in different cell types (McConkey *et al.*, 1988; Baxter *et al.*, 1989; Cotter *et al.*, 1990, 1992; Martin *et al.*, 1990; Waring, 1990; Takano *et al.*, 1991; Bellomo *et al.*, 1992; Nieto *et al.*, 1992). In most cases it is not clear whether an external inducer is required *in vivo* or that cell death is intrinsically programmed in the genome. In some cases cells may be preprogrammed for apoptosis, but external factors are decisive if cells go on with their preprogrammed suicide or are rescued from it. Although the process of apoptosis is presently not completely understood, it seems that a few basic routes to a final common pathway of apoptosis can be discerned. These routes can be categorized on the basis of their susceptibility to inhibitors of protein synthesis such as cycloheximide.

Three basic scenarios have been reported.

(a) Apoptosis is induced by cytotoxic T cells (CTL). It has been shown that well before lysis, DNA in many target cells is fragmented within 5 min after CTL contact (Duke *et al.*, 1983, 1986). In this case *de novo* protein synthesis cannot be involved. Therefore, it seems likely that the CTL can activate a self-destruction pathway in virtually any nucleated target cell. This pathway of apoptosis induction is insensitive to cycloheximide or actinomycin-D. Whether the CTL secretes or injects agents necessary for apoptosis or triggers apoptosis via a membrane-associated molecule is presently not known (Duke & Cohen, 1988; Duke *et al.*, 1989).

(b) Induction of apoptosis requires *de novo* protein synthesis. Therefore, it can be blocked by cycloheximide and related agents. Proteins that are required can be the endonuclease itself, but also activators of a preexisting endonuclease (Alnemri & Litwack, 1989).

(c) Apoptosis is induced by cycloheximide or actinomycin-D treatment. In this pathway, proteins that are necessary to start apoptosis must already be present but are constantly suppressed or inhibited by inhibitory proteins with short half-lives. As a consequence of this, apoptosis-inducing proteins become active when protein synthesis is blocked (Martin *et al.*, 1990; McCall & Cohen, 1991).

#### *Apoptosis in B cells*

During B cell development there are several stages where B cells can undergo apoptosis. First, pre-B cells with re-arranged IgH-genes that produce progeny expressing nonfunctional immunoglobulins are eliminated and probably die via apoptosis (Malynn *et al.*, 1988). Second, at the stage of immature B cells (expressing surface IgM only), those cells that encounter self-antigens die from apoptosis (Cooper & Burrows, 1989; Nemazee & Buerki, 1989a, b; Hartley *et al.*, 1993) in order to delete potentially autoreactive B cell clones. Third, when B cells have passed the stage of clonal abortion, they will express both surface immunoglobulins M and D and localize to the peripheral lymphoid organs. It has been suggested that about 90% of these cells die within 24 h (Freitas *et al.*, 1986) unless they engage antigen and recirculate to the lymph nodes. It has been demonstrated that nuclei of splenic B cells, but not of T cells, contain large amounts of endogenous endonuclease, which can be activated by incubating these nuclei with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (Cohen, 1991). It is thought that this system limits the amounts of virgin B cells within the peripheral B cell pool (Heinen *et al.*, 1988). Fourth, the last stage where B cells can undergo apoptosis is in the follicle during germinal centre reactions. As mentioned above, B cells (centroblasts) in the dark zone of GCs will hypermutate their V-region genes, thereby generating variant antibodies. This is presumably the basis for affinity maturation during immune responses. If the progeny of centroblasts (centrocytes) enter the light zone of GCs they die by apoptosis unless they are rescued by binding to FDCs (Lindhout *et al.*, 1993) (Fig. 8). It is presently not known by which mechanism centrocytes are activated to undergo apoptosis, but preliminary results from our group suggest that protein synthesis is not involved in the activation of apoptosis. On the contrary, it was found that blocking of protein synthesis resulted in high rate apoptosis, suggesting that protein synthesis is part of an apoptosis-inhibiting scenario in these cells and that the killing-machinery is already present in the vast majority of germinal centre B lymphocytes.

Present research is focusing on the nature of signals given by the FDC to prevent apoptosis, on the intracellular pathways involved and on the activation mechanisms of preformed endonuclease in germinal centre B cells.

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